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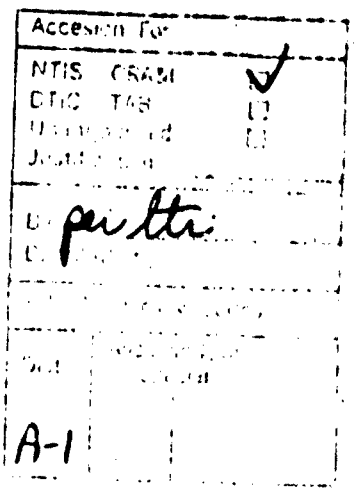
Running Title: Anti-human LFA-1 antibodies cross-react with porcine LFA-1

Monoclonal antibodies against human LFA-1 cross react with porcine LFA-1 and inhibit porcine T-lymphocyte and natural killer cell function[1]



James E.K. Hildreth*, Valerie Holt*,
J. Thomas August*[2], and Mark D. Pescovitz+[3]

From the *Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, and +Immunology Branch, Transplantation Biology Section, National Cancer Institute, Bethesda, MD 20892

Abstract

We have tested five monoclonal antibodies against polypeptides of the human LFA-1/Mac-1/p150-95 glycoprotein family for cross-reactivity with porcine leukocytes. Three monoclonal antibodies against the shared β-subunit were found to react with porcine antigens. One anti-β-subunit monoclonal antibody precipitated the porcine polypeptides corresponding to the α and β subunits of LFA-1 and a polypeptide corresponding to the p150 α-chain seen on human macrophages and granulocytes. Results of two-dimensional electrophoretic studies suggested that the porcine p150 α-subnunit is much more highly acidic than the human equivalent. Two anti-β-subunit antibodies precipitated only the subunits of LFA-1. No polypeptide corresponding to the Mac-1 α-subunit was precipitated by any of the three antibodies. Monoclonal antibodies recognizing the human LFA-1 and Mac-1 α-subunits did not react with pig cells. The anti-β-subunit antibodies inhibited mitogenic and allogeneic stimulation of porcine lymphocytes as well as cytotoxic T cell and natural killer cell-mediated lysis of target cells. Inhibition of cytotoxic T cell function by an anti-β-subunit antibody was found to be additive with blockade by an antibody against the porcine CD8 antigen. The results show that porcine leukocytes express the LFA-1 molecule and that porcine LFA-1 is involved in T cell and NK cell functions as previously shown in human and murine studies.

Footnotes

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2. To whom all correspondence should be addressed.

3. Current address: University of Minnesota, Department of Surgery, Mayo Building, Box 358, Minneapolis, MN 55455.

4. Abbreviations used in this paper: Con A, concanavalin A; CR_3, type three complement receptor; CTL, cytotoxic T lymphocyte; DTH, delayed-type hypersensitivity; FITC, fluorescein isothiocyanate; FMF, flow microfluorometry; GVHD, graft-versus-host-disease; IEF, isoelectric focusing; MAb, monoclonal antibody(s); MLR, mixed-lymphocyte response; NEPHGE, non-equilibrium pH gradient electrophoresis; NK cell, natural killer cell; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

The immune system of swine has emerged as a useful model for human transplantation biology (1). One area of transplantation that has received much attention is the application of monoclonal antibodies (MAb) in the modulation of immune reponses to allografts (2,3) and in the prevention and treatment of graft-versus-host disease (GVHD) (4). For this reason, a large number of murine anti-human lymphocyte monoclonal antibodies (MAb) have been tested for use in the porcine model system, but to date none have been reported to cross-react with porcine lymphocytes. However, we recently developed a panel of anti-porcine MAb which includes MAb that define the porcine T helper and T cytotoxic cells (5,6) and show specificities analogous to the well-characterized human T4 and T8 antibodies. These anti-porcine antibodies have been incorporated into ongoing experiments of porcine bone marrow transplantation as a means of eliminating the GVHD-producing-T cells from bone marrow inocula (Sakamoto et al., manuscript in preparation).

Monoclonal antibodies of particular interest are those directed against the lymphocyte function-associated (LFA-1) antigen. In human and murine systems, these MAb strongly inhibit the mixed lymphocyte response (MLR), mitogen stimulation of T cells, and lysis mediated by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (7-15). Since allograft rejection is suggested to be due to a combination of delayed-type hypersensitivity (DTH) response (MLR-equivalent) and CTL activity in vivo, it is possible that in vivo treatment with anti-LFA-1 reagents would be useful in the control of these rejection responses. In addition, since allogenic resistance to bone marrow engraftment (a phenomenon that has been of major importance in porcine bone marrow transplantation [Popitz et al., manuscript in preparation]) has been associated with NK cell activity in other species (16), MAb against LFA-1 could be of value in overcoming alloresistance.

The LFA-1 molecule is expressed on T and B lymphocytes as well as on macrophages, thymocytes, granulocytes, and a subpopulation of bone marrow cells (7,8,12,17,18). The LFA-1 molecule is composed of two nonconvalently associated polypeptides of Mr 175,000 (α) and 95,000 (β) (7,8,14). Both the human and murine LFA-1 molecules show remarkable structural similarity to a well-characterized macrophage differentiation antigen, Mac-1, which contains an α-subunit of Mr 165,000 and a β-subunit identical to that of LFA-1 (8,18-23). Results of N-terminal sequence analysis of murine LFA-1 and Mac-1 α-subunits suggest that the polypeptides are encoded by duplicated genes and reveal homology with leukocyte (α) interferons (24). A recent study suggests that the α-subunits of LFA-1, Mac-1, and the platelet glycoprotein gpIIb-IIIa are encoded by a single gene (25). MAb against murine and human Mac-1 inhibit binding of C3bi-coated erythrocytes to macrophages, indicating that Mac-1 is closely associated with or represents the type three complement receptor (CR_3) (8,26-28). A third member of the LFA-1/Mac-1 family consisting of an α-subunit of Mr 150,000 and the common β-subunit has been identified (8,18). The function of the p150/93 molecule, which is expressed in high density on macrophages and in low density on monocytes and granulocytes (29), has yet to be defined. Evidence has been presented which suggests that molecules of the LFA-1/Mac-1 family serve as adhesion molecules (14,30-33). Recently, it has been shown that reduced expression of polypeptides of the LFA-1/Mac-1 glycoprotein family results in an immunodeficiency syndrome characterized by poor granulocyte, NK cell, and T cell function (34,35).

We have produced and characterized several MAb against subunits of human LFA-1 and Mac-1 (7,8). The specificities of these MAb were examined by immunoprecipitation from labeled cells and purified antigens. Three MAb reacted with the common Mr 95,000 β-subunit, whereas other MAb reacted with non-cross-reactive epitopes present on the α-subunits of LFA-1 or Mac-1 (8). The MAb against the LFA-1 α-subunit inhibited <u>in vitro</u> T cell function but not

NK cell function or CR_3 activity. The MAb against the Mac-1 α-subunit inhibited CR_3 activity only. Similar results were obtained using MAb against murine LFA-1 and Mac-1 α-subunits (28). In contrast, all functions tested were inhibited by the three MAb against the β-subunit (8).

The conserved use of a polypeptide chain within a species suggested the possibility that subunits of the LFA-1/Mac-1 family might also be antigenically conserved between species. Therefore, we tested anti-human LFA-1/Mac-1 MAb for reactivity against pig cells. In this report we have shown that MAb against the shared β-subunit react with pig leukocytes and immunoprecipitate a β-subunit of Mr 95,000 and 2 distinct α-subunits of Mr 175,000 and 150,000. Moreover, the cross-reactive anti-β MAb inhibit porcine MLR and mitogen responses as well as CTL and NK cell-mediated lysis of target cells. MAb against the α-subunits of LFA-1 and Mac-1 did not react with pig cells.

Materials and Methods

Animals: The inbred miniature swine used in these studies were bred and maintained at the NIH Animal Center at Poolesville, MD. The SLA haplotypes of the animals used in these studies are designated c and d (36). In addition, animals with the recombinant haplotype g, which has the class I genes of the c haplotype and the class II genes of the d haplotype, were used (37).

Monoclonal antibodies: The production of hybridomas secreting MAb against subunits of human LFA-1/Mac-1 has been reported (7,8). H52, H5B9, and MHM.23 (all IgG1, k) recognize the common β-subunit. MHM.24 (IgG1,k) reacts with the α-subunit of LFA-1 and H5A4 (IgG1,k) is specific for the α-subunit of Mac-1. PT4 (74-12-4, IgG2b, k) and PT8 (76-2-11, IgG2a, k) react with porcine T helper and T cytotoxic cells respectively (6). 76-7-4 (IgG2a, k) reacts with a subpopulation of porcine peripheral B cells, and 74-22-15 (IgG1, k) reacts with glass-adherent macrophages (5). 16-1-2 (IgG2b, k), which reacts with murine H-2K^k but not with porcine antigens, was used as a negative control (38). 40D, a murine anti-I-E^k MAb (39), cross-reacts with swine class II MHC antigens (40).

Purification and biotin conjugation of MAb: PT4, PT8, 76-7-4, and 74-22-15 were eluted from protein A Sepharose. H52 was purified on a DEAE column. The fluorescein isothiocyanate (FITC) and biotin conjugations were performed as described (6). The resultant antibodies were titered for staining activity on the FACS II (Becton Dickinson, Sunnyvale, Ca) (data not shown).

Peripheral blood mononuclear cells (PBMC): Pig PBMC were prepared from heparinized whole blood by Ficoll-Hypaque density centrifugation (41).

Immunoprecipitation: Vectorial cell surface iodination and immunoprecipitation were carried out as described previously (8), except that glucose oxidase was used instead of H_2O_2. Immunoprecipitated antigens were analyzed by one-dimensional sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis

(PAGE) using a modification of the method of Laemmli (42), as previously described (8). Two-dimensional isoelectric focusing (IEF)-SDS-PAGE of immunoprecipitated antigens was performed after the method of O'Farrell (8,43). Non-equilibrium pH gradient electrophoresis (NEPHGE) was performed using gels containing 2 percent pH 3.5 to 10 ampholines (44). Antigens were focused for 4 h at 500 volts.

Dual-parameter flow microfluorometry (FMF) analysis: FMF was performed as previously described (6). PBMC were reacted first with H52-FITC. The cells were washed with Hanks' balanced salt solution containing 5 percent normal pig serum (GIBCO, Grand Island, NY), 0.1 percent Na azide, and 0.1 percent bovine serum albumin, and then reacted with biotin-conjugated second antibody. The cells were washed and stained with Texas Red-Avidin. The intensity of red and green fluorescence was determined on the FACS II (Becton Dickinson, Sunnyvale, CA) using computer-generated contour plots. Single-parameter analysis with H52-FITC was performed in a similar manner except that the second antibody was not added.

Proliferative assays: PBMC were suspended at 4 X 10^6 cells/ml in RPMI 1640 supplemented with 8 percent fetal pig serum, 1 mM glutamine, 1 mM Na pyruvate, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 µg/ml steptomycin, 50 µg/ml gentamicin, and 2 X 10^{-5} M 2-mercaptoethanol (referred to from this point as "medium"). Responder cells, 0.1 ml at 4 X 10^6/ml, and irradiated (2000 rad) stimulator PBMC, 0.1 ml at 4 X 10^6 cells/ml, were incubated for five days in 6 percent CO_2 in humidified air. Phytohemagglutinin (PHA), Form M (Gibco, Grand Island, NY) at a final dilution of 1:400, or concanavalin A (con A), (Sigma, St. Louis, MO), at a final concentration of 10 µg/ml, was added and the cells were incubated for an additional 2 days. One µCi of ^{3}H-thymidine was added for the final 6 h of the culture period. The wells were harvested with the PHD cell harvester (Cambridge Technologies, Cambridge, MA). Assays were done in replicates of 3 or 5 in 96-well plates (Falcon,

Becton Dickinson, Oxnard, CA). Inhibition assays were performed with the appropriate antibody in 0.01 ml of medium, added at the initiation of culture or at the indicated time point thereafter.

Cell-mediated lymphocytotoxicity: PBMC were incubated as in the proliferative assays described above, but for 7 days in culture flasks (Costar, Cambridge, MA). The cells were then harvested and counted. Antibody blocking studies were conducted by adding the effector cells, at various densities in a volume of 10 µl, to 96 well plates, to which the appropriate antibody (10 µl ascites fluid diluted 1:50) was then added. The plates were incubated for 15 min, after which were added 10^4 ^{51}Cr-labeled 48 h PHA blasts in a volume of 100 µl. The supernatants were harvested after 6 h incubation using the Titertek harvesting system. Percent killing was determined using the formula:

$$\text{percent specific lysis} = \frac{(\text{cpm experimental} - \text{cpm spontaneous})}{(\text{cpm maximal} - \text{cpm spontaneous})} \times 100$$

Maximal release was determined by treating targets with 0.1 ml of 1 N HCl.

Natural killing assays: The method of Kim was used (45). Briefly, 2 X 10^6 PBMC were incubated overnight in medium with 10 percent fetal calf serum (instead of fetal pig serum), in 6 percent CO_2. The cells were plated as for the CTL assays including the addition of blocking antibody. Human K562 erythroleukemia cells labeled with ^{51}Cr were used as targets. The plates were incubated for 6 h, harvested, and the percent lysis calculated as for the CTL assay.

Results

Antibodies against the human β-subunit cross-react with pig lymphocytes

Monoclonal antibodies against subunits of the human LFA-1/Mac-1 glycoprotein family (8) were initially tested for cross-reactivity with pig leukocytes by screening the MAb on cryostat sections of pig spleen. Three MAb that recognized epitopes on the common β-subunit of the human glycoproteins (H52, H5B9, and MHM.23) reacted with pig leukocytes, whereas the MAb recognizing the LFA-1 α-subunit (MHM.24) and the Mac-1 α-subunit (H5A4) did not (data not shown).

The results obtained in immunohistologic studies were confirmed by immunoprecipitation from ^{125}I-surface-labeled pig PBMC. Each of the 3 MAb against the human β-subunit precipitated porcine polypeptides corresponding in Mr to the LFA-1 α- and β-subunits (Figure 1). The MHM.23 MAb also consistently precipitated a polypeptide corresponding to the human p150 subunit; this polypeptide was only weakly represented in precipitates using the H52 and H5B9 MAb. These results correlate with the observation that the H52 and H5B9 MAb recognize the same epitope on the human β-subunit, whereas the MHM.23 MAb recognizes a distinct β-subunit epitope (Hildreth, unpublished results). It is possible that binding of H52 and H5B9 cause dissociation of the p150 subunit from the β-subunit whereas binding of MHM.23 does not, or that there are multiple β-subunits (46). No polypeptides were precipitated by the MHM.24 or H5A4 MAb, which recognize the α-subunit of human LFA-1 and Mac-1, respectively (Figure 1).

Two-dimensional analysis of pig LFA-1 antigens

Pig and human LFA-1 antigens were compared by IEF and SDS-PAGE two-dimensional analysis. Immunoprecipitation from human PBMC using the anti-β subunit antibodies yielded 4 distinct polypeptide bands: the Mr 165,000 Mac-1 α-subunit, Mr 175,000 LFA-1 α-subunit, p150 α-subunit, and the

common Mr 95,000 β-subunit (Figure 2B). In contrast, two-dimensional analysis of anti-β-subunit precipitates from pig PBMC showed only bands corresponding in pI and molecular weight to the common β-subunit and the LFA-1 α-subunit (Figure 2a). A polypeptide corresponding to the Mac-1 α-subunit was not precipitated from pig PBMC by any of the anti-β-subunit MAb. In spite of the presence of a strong p150 band in single-dimensional SDS-PAGE analysis of MHM.23 immunoprecipitates from pig PBMC, this band was also consistently absent when the precipitated antigens were analyzed by two-dimensional IEF-SDS-PAGE (Figure 2A).

Additional experiments were conducted to determine if the apparent absence of the Mac-1 α-subunit could be attributed to a lack of the appropriate cell population. The Mac-1 antigen of murine and human cells is expressed on granulocytes, monocytes, NK cells, and a small subpopulation of T cells (14,47). A recent study has shown the human p150 subunit is expressed in high density on macrophages and in low density on monocytes and granulocytes (29). Thus, the precipitation of p150 from pig PBMC suggested that monocytes and/or granulocytes were present in the labeled cells. The failure to precipitate a Mac-1 equivalent α-subunit therefore did not appear to be due to a lack of the appropriate cells in the pig PBMC preparations. However, to test the possibility that pig Mac-1 was expressed only on mature macrophages, pig PBMC were cultured for 5 days at 5 X 10^6 cells/ml, and immunoprecipitation from extracts of adherent (macrophages) and non-adherent cells was performed with the Mac-1 α-subunit-specific MAb (H5A4) and the anti-β MAb MHM.23. No bands were precipitated from either cell type by the anti-Mac-1 α-subunit-specific MAb (Figure 3). The MHM.23 immunoprecipitates demonstrated that the p150 molecule was the predominant α-subunit expressed on pig macrophages (Figure 3). However, two-dimensional analysis of the MHM.23 precipitates from adherent and non-adherent cells again showed only the LFA-1 α-subunit and the β-subunit (data not shown).

Analysis of the p150 subunit by non-equilibrium two-dimensional analysis

Failure of the pig p150 molecule to focus in IEF gels suggested the possibility that this polypeptide has a pI beyond the pH range of the gels. We therefore analyzed the pig antigens immunoprecipitated by MHM.23 MAb in a non-equilibrium pH gradient electrophoresis (NEPHGE) system (44). MHM.23 MAb immunoprecipitates from human and pig PBMC were focused for a total of 2000 volt-hours with the basic buffer at the top of the focusing gels. NEPHGE analysis of MHM.23-precipitated antigens from human cells showed each of the 4 subunits normally present in two-dimensional IEF-SDS-PAGE analysis at a pH 1 to 2 units more basic than their respective isoelectric points (Figure 4). NEPHGE analysis of MHM.23 immunoprecipitates from pig PBMC showed the LFA-1 α-subunit and the β-subunit, as seen in IEF studies, and in addition a weak polypeptide band of Mr 150,000 on the acidic end of the gel (Figure 4). These results suggest the possibility that the pig p150 subunit is a much more highly acidic molecule than its human equivalent. No band corresponding in molecular weight to the Mac-1 α-subunit was seen in the pig NEPHGE gels.

Flow microfluorimetry (FMF) analysis of LFA-1 β chain distribution

Human LFA-1 is expressed at high densities on T cells and at lower densities on B cells (7,17). Single-parameter FMF analysis of porcine PBMC with the anti-β subunit MAb H52 showed that all peripheral blood lymphocytes expressed the LFA-1 marker (Fig. 5). The level of expression was bimodal, as seen with human lymphocytes (7,17), with some cells expressing high levels (bright) and other cells expressing low levels (dull) of LFA-1.

The distribution of LFA-1 expression was further analyzed by dual-parameter FMF with FITC-labeled H52 and other MAbs that are known to react with porcine T cells (PT4, PT8), B cells (76-1-4), or macrophages (72-22-15) (Fig. 6). It was found that the bimodal distribution seen in the single-parameter analysis was not random. In particular, a ' of the macrophages which express the two β-subunit-associated polypeptide (LFA-1 α

and p150) were contained in the H52 bright population. The majority of the B cells (76-7-4-positive) were contained in the H52 dull population. The helper ($PT4^+$) and the cytotoxic/suppressor ($PT8^+$) T cells were represented in both populations. The PT8 dull population, which has been previously shown to contain the PT4/PT8 dual-expressing cells (6), was restricted to the H52 dull cells.

Effect of LFA-1 on in vitro proliferative responses

LFA-1 antibodies have been shown to block the proliferative responses of lymphocytes to both alloantigens and mitogens in mouse and man. Similarly, the anti-β-subunit MAb H52 completely inhibited the porcine allogeneic mixed lymphocyte response (Figure 7). Inhibition by H52 was greater than that by PT4 MAb. In other experiments (not shown), this level of inhibition by PT4 was found to be maximal. The inhibition by both PT4 and H52 affected an early stage of T cell antigen-induced proliferation, since the antibodies failed to block if added at any time after the first 24 to 48 hours (Fig. 7).

The anti-LFA-1 also inhibited the proliferative response to mitogenic stimuli (Table I). Nearly complete inhibition of the proliferative response was observed with suboptimal doses of Con A. The response to PHA was generally less sensitive to blockade by H52 than the response to Con A, but at suboptimal doses of PHA, inhibition was nearly complete. PT4 also inhibited the mitogen response, although to a variable and lesser degree than that caused by anti-LFA-1. MAb 16-1-2 and 74-22-18, included as isotype controls, had no effect on the porcine mitogen responses.

Effect of LFA-1 on in vitro CTL activity

The FMF analysis indicated that PT8-positive cells were LFA-1 positive. By analogy with both human and murine results, anti-porcine LFA-1 antibodies would be expected to inhibit CTL activity. This hypothesis was tested with an in vitro CTL assay. Class I MHC-specific killing was examined using the SLA combination dd anti-cc for the MLR and testing the resultant CTL on gg

targets. The inhibitory antibodies were incubated with the effector cells for 15 min prior to the addition of the target cells. Under these conditions, lysis was not inhibited by either of two control antibodies (74-22-15, IgG1, or 400, IgG2) (Fig. 8). As seen previously (6), PT8 blocked the CTL-mediated killing. The anti-LFA-1 antibody blocked CTL killing to even a greater degree than did PT8. The level of inhibition was at plateau for both antibodies, as determined by titering the concentration of antibody at a fixed effector: target ratio (data not shown).

Although the inhibition of CTL-mediated lysis by anti-LFA-1 was profound, it was not complete even at saturating MAb concentrations. This suggested that some CTL were not susceptible to blockade by this antibody. To test this hypothesis, both PT8 and H52 were added separately and together as inhibitors of CTL-mediated lysis. Figure 9 again shows that neither PT8 nor H52 alone completely inhibited lysis. When a mixture of both PT8 and H52 were added to the assay, CTL inhibition was complete.

Effect of anti-LFA-1 antibody on natural killer cell function

Porcine natural killer cells have been previously characterized by the ability of either fresh or overnight cultured PBL (without any stimulator cells) to mediate lysis of the human erythroleukemia cell line K562. The cell surface phenotype of these porcine NK cells is unknown and is currently under investigation. Since all PBMC in pigs expressed the β-subunit of the LFA-1 antigen, it was assumed that this antigen would also be present on the NK cells.

The H52 MAb inhibited NK cell-mediated lysis of K562 cells almost completely (Fig. 10). In contrast, neither PT8 nor PT4 was able to block lysis of K562 by NK cells. Moreover, there was no additive inhibition of killing with a combination of H52 and PT8. These results indicate that NK cells in the pig express the LFA-1 subunit and that their killing of target cells involves the LFA-1 molecule β-subunit.

Discussion

The pig has become an important model for studying the biology of human transplantation and experimental methods of immune modulation (1,36,37). Monoclonal antibodies have recently been used to reverse rejection phenomena in human transplant recipients (2,3), and represent an important tool for immunomodulation. MAb against porcine lymphocyte antigens are thus of considerable interest because of the potential for studying their in vivo effects in an animal model. Monoclonal antibodies against human and murine LFA-1 inhibit CTL and NK cell function, as well as the mitogenic and antigenic stimulation of T cells (7,8,14). These MAb are therefore of potential use in reversing allograft rejection and preventing GVH reactions. For these reasons we have screened a panel of MAb against the human LFA-1/Mac-1 glycoprotein family for cross-reactivity with porcine leukocytes.

Five previously reported MAb against subunits of the human LFA-1/Mac-1 family (8) were tested for binding to pig antigens. Cross-reaction was observed for all three MAb that recognize the common Mr 95,000 β-subunit but not for MAb recognizing the α-subunits of LFA-1 and Mac-1 molecules. Similar results were obtained when the 5 MAb were tested against monkey leukocytes (J. Hildreth, unpublished). These results suggest that the common β-subunit is highly conserved between species whereas the α-subunits are not. However, it is possible that the MAb against the α-subunits recognize non-conserved epitopes.

The pig LFA-1 molecule consisted of subunits of Mr 175,000 and Mr 95,000 when analyzed under reducing conditions, with isoelectric points in the pH 5.5 to 6.3 range. These results are identical to those obtained for murine and human LFA-1 (8,14) and suggest that the LFA-1 molecule is a highly conserved bimolecular complex. A polypeptide of Mr 150,000 (p150) was also precipitated from pig PBMC by the anti-β MAb and probably represents the pig equivalent of

the human LeuM5 macrophage/granulocyte antigen. Unlike the human p150 molecule, the pig p150 did not focus in two-dimensional IEF-SDS-PAGE gels. However, a weak band of Mr 150,000 was observed in NEPHGE experiments at the acidic end of the gels, suggesting that the pig p150 is a highly acidic protein. Immunoprecipitates from pig PBMC were notable for the absence of a polypeptide band corresponding in size and pI to the murine and human Mac-1 α-subunit. This was true even when pig cells were enriched for macrophages from which the anti-β MAb precipitated strong β and p150 bands. There are several possible explanations for this finding, including: (1) binding of anti-β-subunit MAb causes dissociation of the pig Mac-1 α-subunit; (2) a second antigenically distinct pig β-subunit is associated with the Mac-1 α-subunit; (3) the CR_3 function in the pig may be served by one or more proteins other than Mac-1.

The cellular distribution of the LFA-1 antigen (β-subunit) on porcine PBMC has been examined by FMF. The fluorescence intensity of this cell population stained with an anti-β-subunit MAb showed a bimodal distribution similar to that previously observed in murine and human studies (7,17). All cells expressed the antigen, but a small population expressed four times as much antigen as the remaining cells. The availability of MAb that react with distinct porcine subsets and the technology of dual-parameter FMF analysis allowed us to examine which particular cell phenotypes fell within the biomodal LFA-1 distribution. Pig B cells, the majority of which were marked with an anti-CD1 antibody 76-7-4 (5 and M. Pescovitz et al., in preparation), were contained entirely within the dull population. This has been seen with human B cells as well (7). Both T helper and T cytotoxic cells are split between bright and dull populations, as previously observed for human T cells (Hildreth, unpublished). It is unknown whether the bright/dull division in the T cells marks cells with distinct functional phenotypes. Results of a previous study do suggest, however, that increased expression of LFA-1

correlates with increased T cell effector function (48). Macrophages, as defined by the MAb 74-22-15, are entirely LFA-1-bright. The macrophage level of expression can not be explained solely by size, since porcine macrophages are only slightly larger than lymphocytes as determined by light scatter on the FMF analyzer (data not shown). One explanation is that the higher expression on the macrophage resulted from higher expression of the LFA-1 β chain. In humans the β chain would also be associated with Mac-1, LFA-1, and p150 α-chains on the macrophage cell surface (8,12,18). As shown above, there is no evidence of a porcine Mac-1 α-chain. However, purified porcine macrophages appear to express high levels of the p150 α-chain as well as the LFA-1 α-chain. It is therefore likely that pig macrophages express the β-subunit at higher levels than lymphocytes, since on lymphocytes the β-subunit is associated only with the LFA-1 α-subunit. The final resolution of whether porcine macrophages express Mac-1 as well as the LFA-1 and p150 α-chains awaits the generation of porcine α-chain specific antibodies.

Various in vitro assays were used to examine the effect of anti-β subunit MAb on porcine lymphocyte function. Both MHC antigen-specific and mitogen-induced lymphocyte proliferation were blocked by anti-LFA-1 antibodies. The inhibition could be partially overcome by high concentrations of PHA or Con A, as previously shown in human studies (8,49). Time course studies of the inhibition of mixed lymphocyte reactivity indicated that LFA-1 blocked at an early stage; no inhibition resulted if addition of antibody was delayed for 48 hours, and maximal inhibition was seen only in the first 6 to 8 hours. These results suggest that the MAb are not simply toxic to the cells, and are consistent with the postulated role of the LFA-1 molecule in early cell-cell interactions required for lymphocyte activation (14,30,31). The anti-LFA-1 antibodies also profoundly inhibited CTL-mediated lysis of target cells. An antibody (PT8) to the porcine CD8 antigen also blocked CTL-mediated lysis, but much less well than did the anti-LFA-1 MAb. Inhibition by PT8 and

LFA-1 antibodies was additive, and complete inhibition was observed when the antibodies were used together. A similar additivity between CD8 and LFA-1 antibodies has been reported previously in man (14,49). This additive inhibition by PT8 and LFA-1 indicated that all CTL were subject to MAb blockade, but that either clones used only CD8 or LFA-1 as association molecules or that some clones required both CD8 and LFA-1. Results of a recent study using CTL clones and MAb supports the use by CTL of either LFA-1 or CD8 as the molecule mediating conjugation (50).

Although porcine NK cells have been studied for a number of years, MAb reactive with them have only recently been described (51). Anti-LFA-1 MAb have been shown to block both human and murine NK cell activity (7,8,14). Similarly, in the present study porcine NK cell-mediated lysis of K562 target cells was almost completely blocked by the anti-LFA-1 antibodies. Porcine NK cell activity was not blocked by anti-porcine CD8 MAb, indicating that lysis was mediated by a cell type distinct from the classic CTL. Therefore, like CTL, porcine NK cells express and appear to require LFA-1 for function.

We have shown that pig leukocytes express the LFA-1 molecule and that MAb against this structure are highly immunosuppressive in the in vitro porcine system. Allograft rejection is a complex phenomenon probably involving a number of different T cell types and stimulatory signals (3,50). Since anti-LFA-1 MAb are able to block both the induction and effector phases of T cell activation as well as NK function, they may prove useful as immunosupressant reagents in clinical transplantation. Studies are currently underway to evaluate the efficacy of these MAb for suppression of allograft rejection using the pig as animal model.

TABLE I

Effect of anti-LFA-1 monoclonal antibody on mitogen-induced proliferation of porcine PBMC[a].

Inhibiting MAb	Mitogen		
	None	PHA[b]	Con A[c]
None	818 ± 43[d]	155836 ± 10269	57115 ± 3269
H52	1084 ± 159	115459 ± 10031 (25.9)[e]	2729 ± 279 (95.2)
PT4	1200 ± 87	123770 ± 9778 (20.5)	34859 ± 3766 (38.9)
16-1-2	1187 ± 48	208324 ± 3198 (-33.7)	77000 ± 6210 (-34.8)
74-22-15	749 ± 14	186449 ± 8529 (-19.6)	79768 ± 17789 (-39.7)

[a] gg PBMC were incubated for 48 hr in the presence of a 1:200 final dilution of MAb ascites with or without mitogen. 1 μCi of ^{3}H-thymidine was added to each well for the final 6h of culture.

[b] PHA (Form M, Gibco) was used at a dilution of 1:400.

[c] Con A was used at a final concentration of 10 μg/ml.

[d] Mean total ^{3}H-thymidine incorporation (cpm) ± standard error for triplicate samples.

[e] Percent inhibition of mitogenic stimulation is shown in parentheses.

Figure Legends

Figure 1. Anti-human LFA-1/Mac-1 immunoprecipitation from pig PBMC. Pig PBMC were surface-labeled with ^{125}I and then extracted with a buffer solution containing 0.5 percent NP-40. Antigens from 10^7 cell equivalents of extract were immunoprecipitated with 100 µl of ascites fluid diluted 1:100. Precipitates were subjected to 7.5 percent SDS-PAGE under reducing conditions and autoradiography with an enhancing screen.

Figure 2. Two-dimensional IEF-SDS-PAGE analysis of pig and human LFA-1 antigens. Pig and human PBMC were labeled and extracted as described for Figure 1. Antigens were precipitated with MHM.23 ascites diluted 1:100 from pig (A) and human (B) PBMC and analyzed by two-dimensional IEF-SDS-PAGE. Polypeptide bands were visualized by autoradiography with enhancing screens. The pH range is shown at the top of the figure. Lα = LFA-1 α-subunit; Mα = Mac-1 α-subunit; p150 = Mr 150,000 α-subunit; β = β-subunit.

Figure 3. Immunoprecipitation of LFA-1 and p150-95 from 5 day-cultured pig PBMC. Pig PBMC were cultured in RPMI-1640 supplemented with 10 percent fetal bovine serum at 5 X 10^6/ml for 5 days. Non-adherent and adherent cells were isolated and surface labeled, then extracted with detergent. Antigens were immunoprecipitated with a 1:100 dilution of MHM.23 ascites and subjected to 7.5 percent SDS-PAGE. Polypeptides were visualized by autoradiography with enhancing screens. A = H5A4 (anti-Mac-1 α), B = MHM.23 (anti-β), Adh = Adherent cells, Non Adh = Non-adherent cells.

Figure 4. Non-equilibrium pH gradient electrophoresis (NEPHGE) of anti-LFA-1 β-subunit immunoprecipitates from pig and human PBMC. Cells were labeled and extracted as described for Figure 1. Antigens were immunoprecipitated with a 1:100 dilution of MHM.23 ascites, then analyzed by NEPHGE and 7.5 percent SDS-PAGE as described in Materials and Methods. Polypeptides were visualized by autoradiography with an enhancing screen. Arrows indicate polypeptides of Mr 150,000.

Figure 5. Single-parameter FMF analysis of LFA-1 β-subunit expression on porcine PBMC. Whole PBMC were stained with H52-FITC prior to analysis by FMF with logarithmic amplification, as described in Materials and Methods. The shaded area represents the background fluorescence of unlabeled cells.

Figure 6. Dual-parameter FMF analysis of LFA-1 β-subunit expression versus expression of other antigens on porcine PBMC. Whole PBMC were stained with H52-FITC followed by the second biotinylated antibody and Texas Red-Avidin. The relative intensity of green fluorescence (LFA-1 β-subunit) is shown on the x axis, and red fluorescence (second MAb) on the y axis. The cell number is represented by the contour lines, which are drawn at 10-, 20-, 40-, and 80-cell levels. 76-7-4 is a marker for peripheral B cells; 74-22-15 for macrophages; PT4 for T helper cells; and PT8 for T cytotoxic cells.

Figure 7. Time course of anti-LFA-1 inhibition of porcine mixed lymphocyte response. H52 (O) or PT4 (Δ) was added to a cc anti-dd MLR at the times indicated. MAb was used in the form of ascites fluid at a final dilution of 1:100. Proliferation (total cpm incorporated minus cpm incorporated in the autologous MLR) at 120 h following

initiation of culture is plotted. The shaded area is the mean ± 1 SEM of the control MLR in the absence of inhibitor or in the presence of an irrelevant MAb.

Figure 8. Effect of LFA-1 on CTL activity. Class I-specific CTL were tested for lytic activity against PHA blasts in the presence of various MAb as described in Materials and Methods. MAb (ascites fluid) were added to a final dilution of 1:100. Antibodies used were against porcine macrophages (74-22-15; □), swine class II MHC (40 D; ▲), porcine T8 (PT8; △), and LFA-1 (H52; O). Lysis in the absence of MAb (●) is also shown.

Figure 9. Additive inhibition of CTL killing by PT8 plus LFA-1. Class I-specific CTL at a 100:1 effector:target ratio were tested for lytic activity in the presence of either PT8 or H52 alone or in combination. Effector cells were pre-incubated with MAb before adding target cells as described in Materials and Methods. Final concentration of each MAb was constant (ascites fluid at final dilution of 1:100).

Figure 10. Inhibition of NK-mediated lysis by anti-LFA-1 MAb. The lytic activity of porcine PBMC on the NK target K562 was tested in the presence of either PT8, H52, PT8 + H52, or PT4 as described in Materials and Methods. The final concentration of each MAb was constant (ascites fluid at final dilution of 1:100). Effector:target ratio was 100:1.

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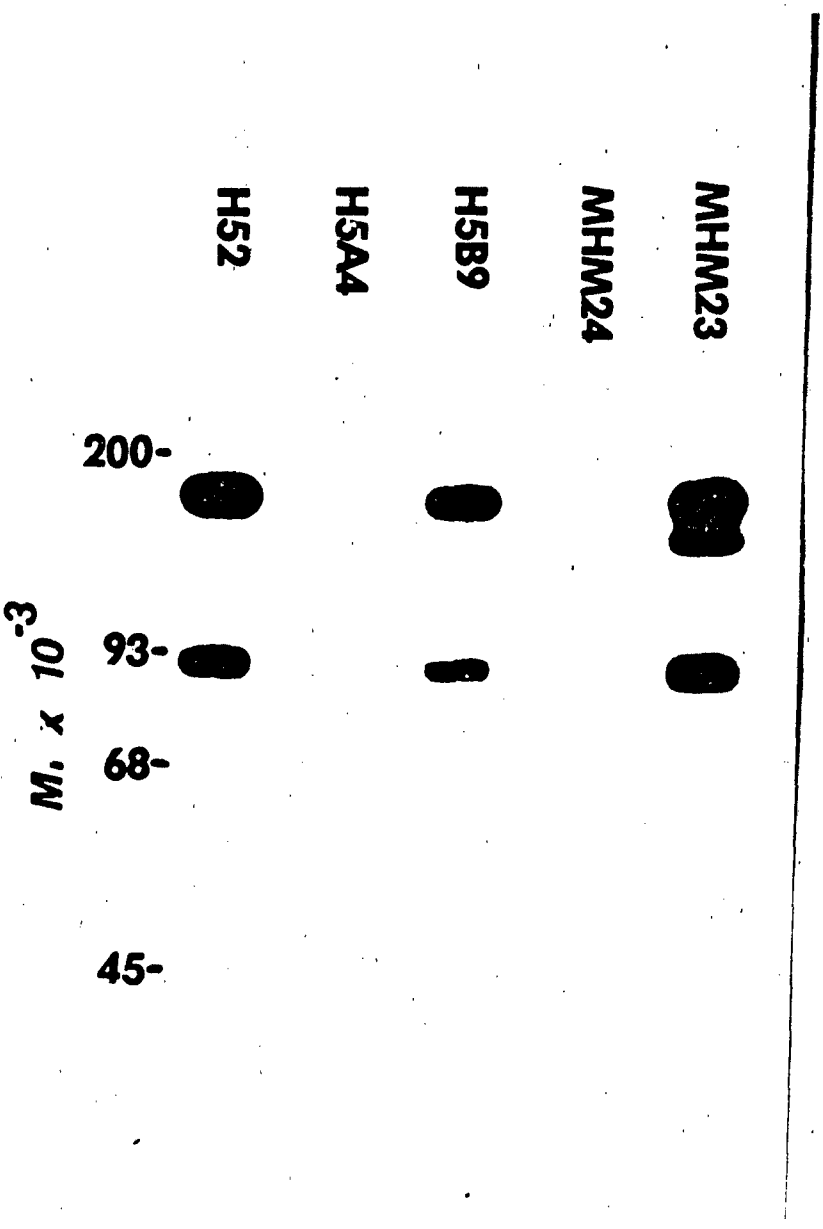
FIGURE 1



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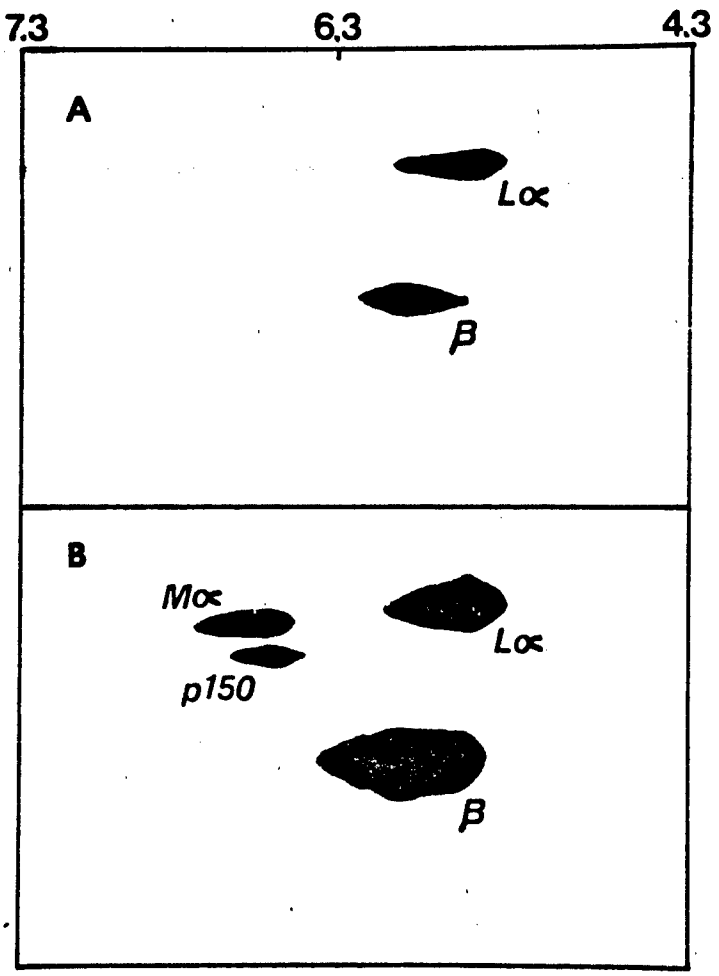

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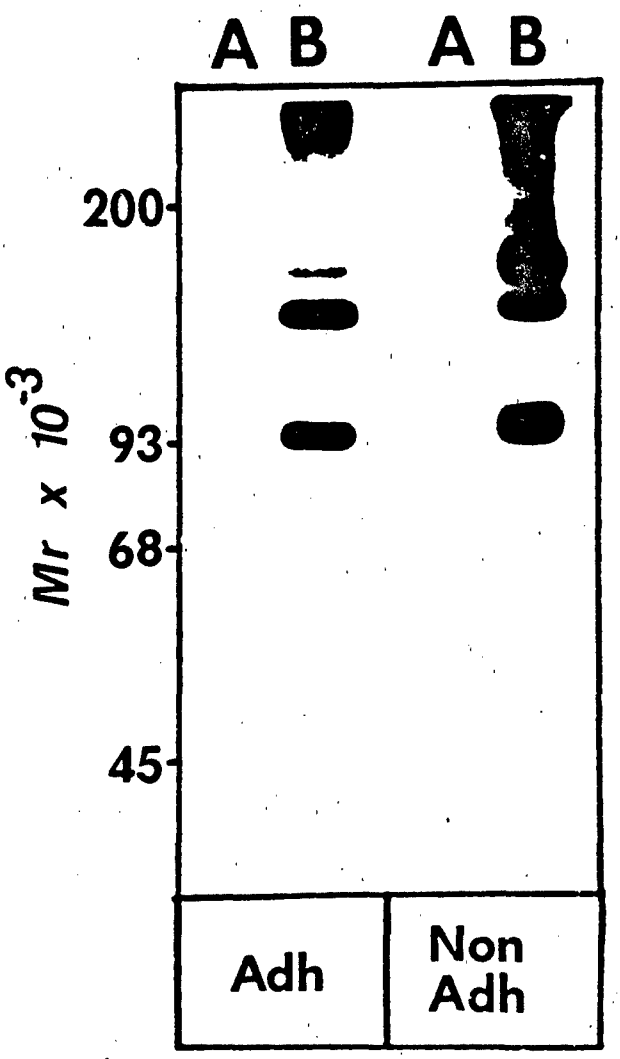

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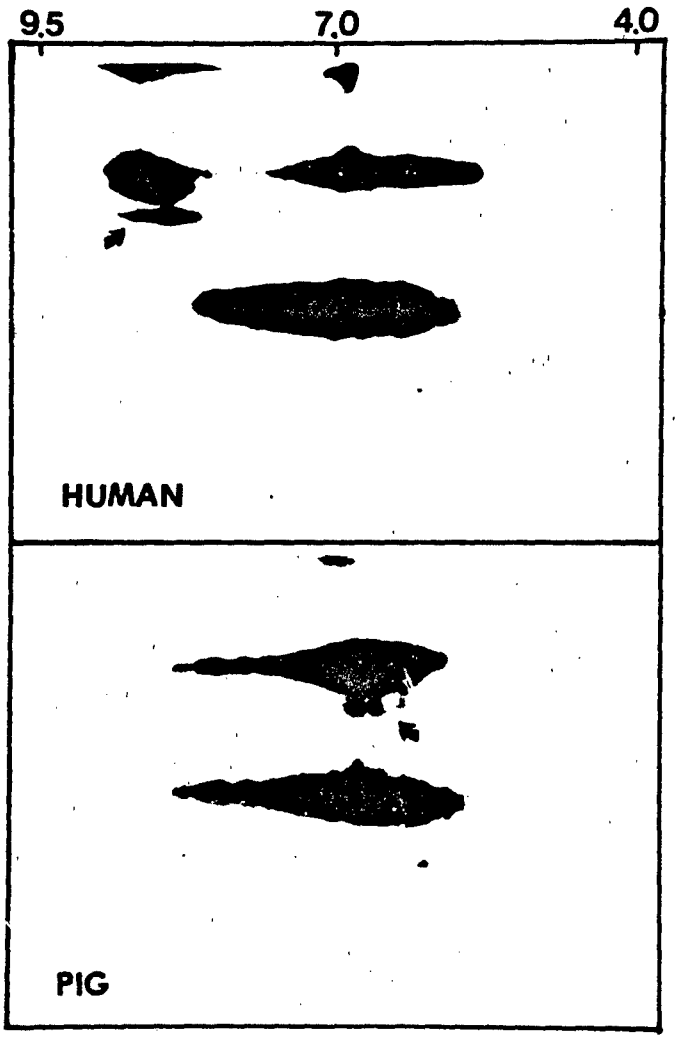

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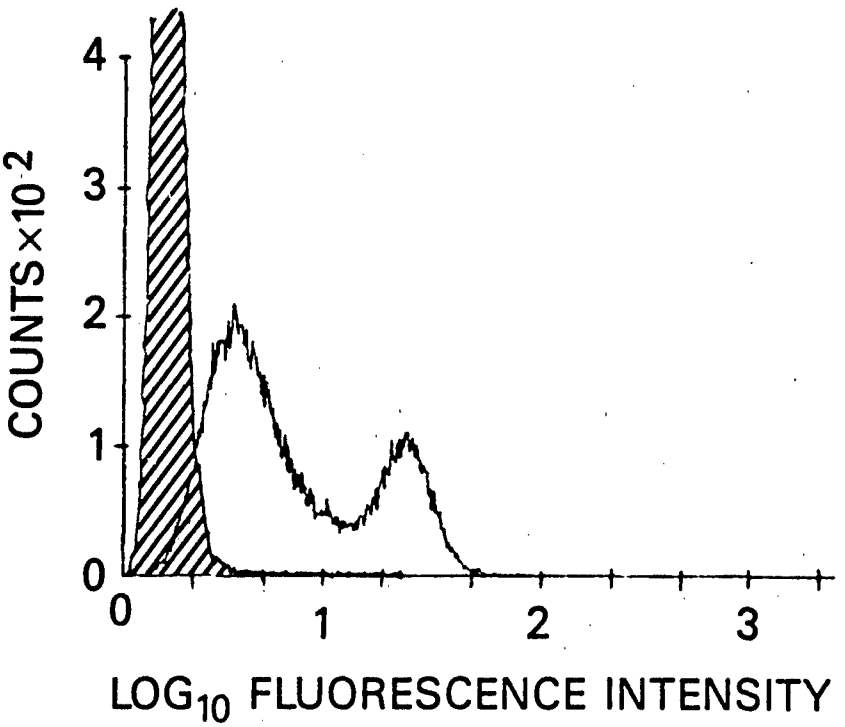

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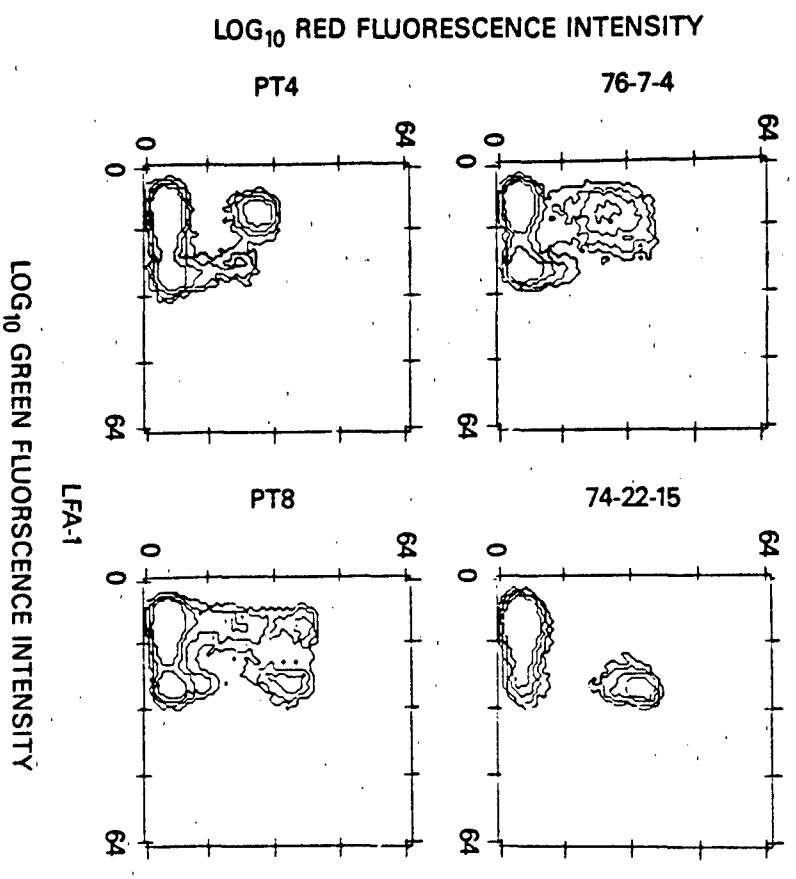
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FIGURE 7

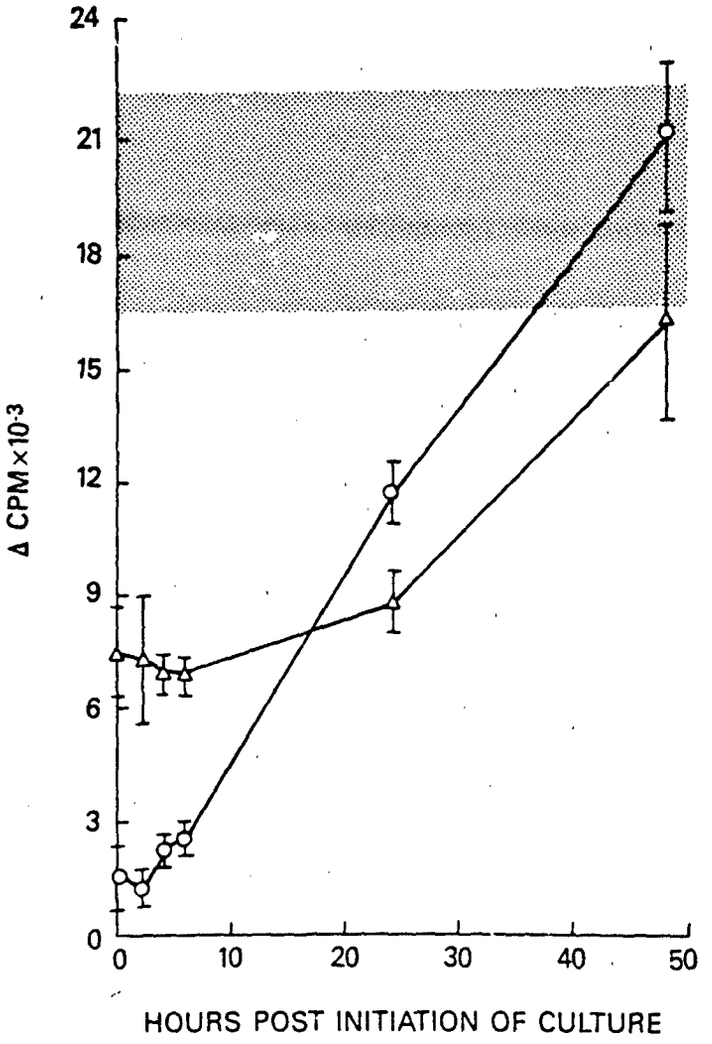
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FIGURE 8

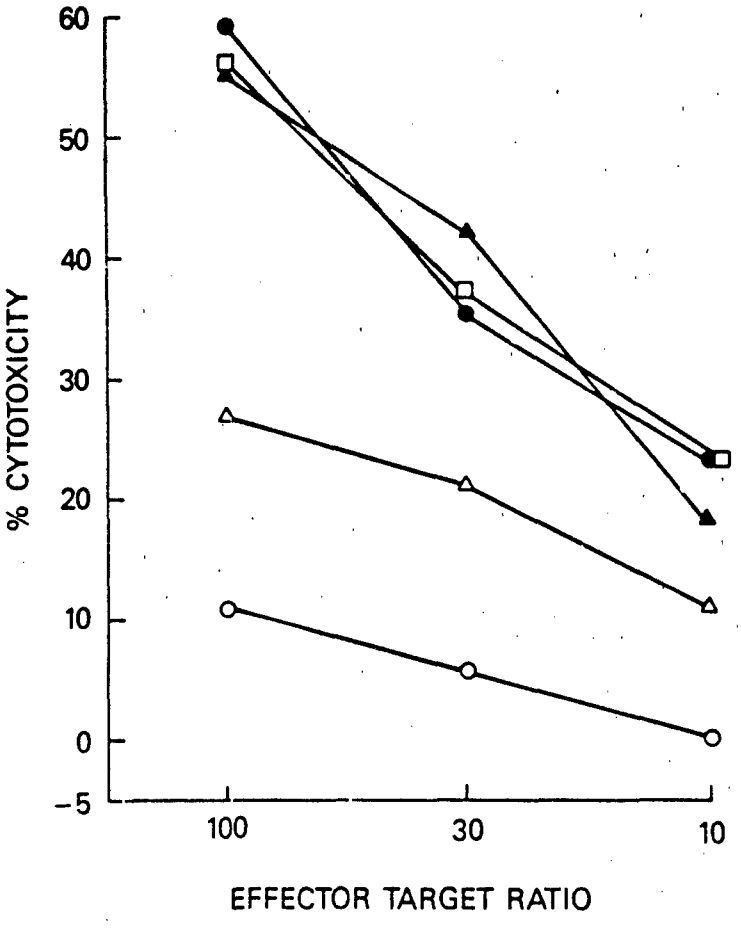
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FIGURE 9

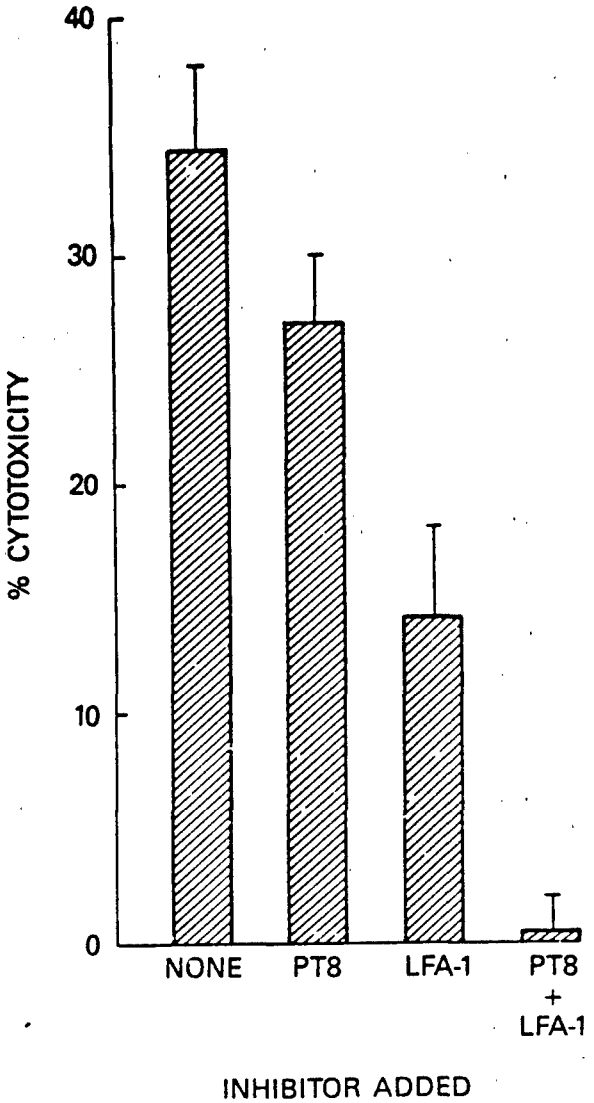
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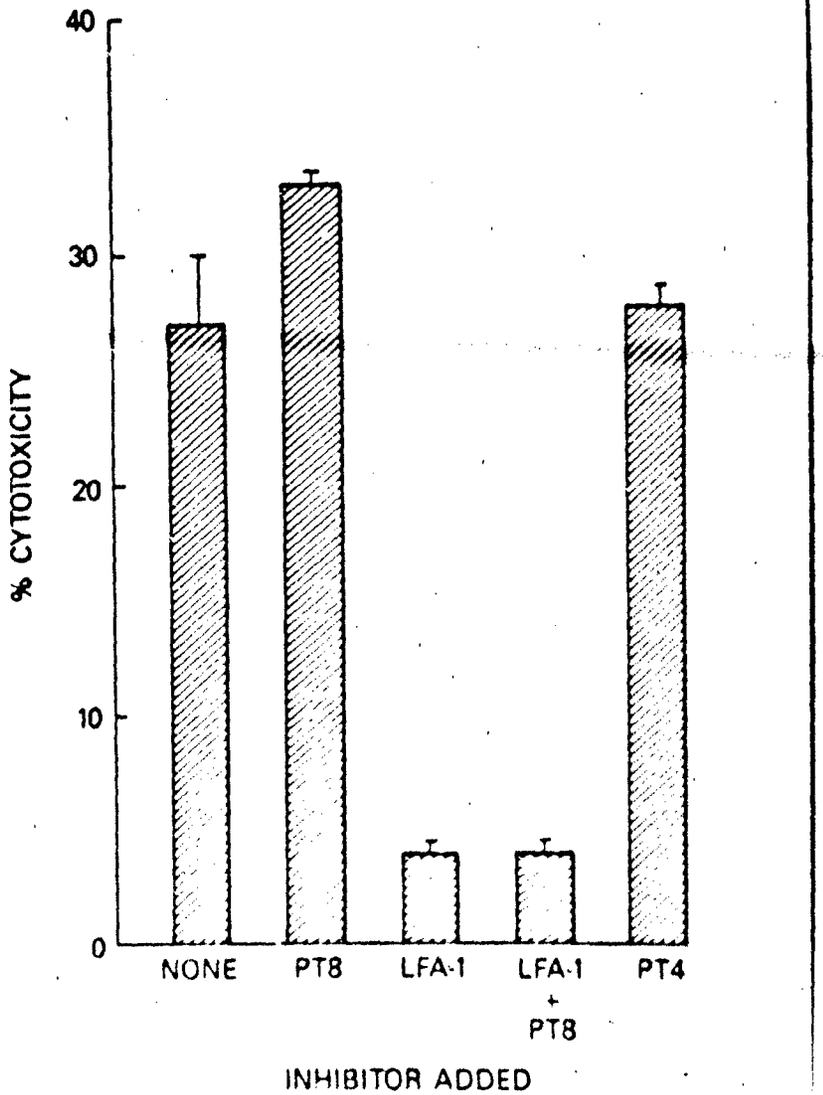
FIGURE 10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